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(54) **Uses of recombinant colony stimulating factor-1 for treatment of leukopenia**

(57) Recombinantly produced colony stimulating factor 1 (CSF-1) is useful in the form of a medicament for the treatment of leukopenia including enhancement of white blood cell count.

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**Description**Technical Field

**[0001]** The present invention relates to the use of recombinantly produced human colony stimulating factor-1 (CSF-1) in the treatment of viral infectious disease in mammals, the virus being cytomegalovirus. The invention also relates to the use of such CSF-1 in the treatment of leukopenia and in enhancing white blood cell count.

Background Art

**[0002]** The ability of certain factors produced in very low concentration in a variety of tissues to stimulate the growth and development of bone marrow progenitor cells into granulocytes and/or macrophages has been known for nearly 15 years. The presence of such factors in sera, urine samples, and tissue extracts from a number of species is demonstrable using an *in vitro* assay which measures the stimulation of colony formation by bone marrow cells plated in semi-solid culture medium. There is no known *in vivo* assay. Because these factors induce the formation of such colonies, the factors collectively have been called Colony Stimulating Factors (CSF).

**[0003]** More recently, it has been shown that there are at least four subclasses of human CSF proteins which can be defined according to the types of cells found in the resultant colonies. One subclass, CSF-1 results in colonies containing macrophages predominantly. Other subclasses produce colonies which contain both neutrophilic granulocytes and macrophages; which contain predominantly neutrophilic granulocytes; and which contain neutrophilic and eosinophilic granulocytes and macrophages.

**[0004]** There are murine factors analogous to the above human CSFs, including a murine factor called IL-3 which induces colonies from murine bone marrow cells which contain all these cell types plus megakaryocytes, erythrocytes, and mast cells, in various combinations. These CSFs have been reviewed by Dexter, T. M., *Nature* (1984) 309:746, and Vadas, M. A., et al, *J Immunol* (1983) 130:793, Clark, S.C., *Science* (1987) 236:1229, and Sachs, L., *Science* (1987) 238:1374.

**[0005]** The invention herein is concerned with the use of recombinant proteins which are members of the first of these subclasses, CSF-1. This subclass has been further characterized and delineated by specific radioimmunoassays and radioreceptor assays --e.g., antibodies raised against purified CSF-1 are able to suppress specifically CSF-1 activity, without affecting the biological activities of the other subclasses, and macrophage cell line J774 contains receptors which bind CSF-1 specifically. A description of these assays was published by Das, S. K., et al, *Blood* (1981) 58:630.

**[0006]** A significant difficulty in putting CSF proteins in general, and CSF-1 in particular, to any useful function has been their unavailability in distinct and characterizable form in sufficient amounts to make their employment in therapeutic use practical or even possible. These deficiencies may be remedied by providing purified human and murine CSF-1 in useful amounts through recombinant techniques.

**[0007]** (A CSF protein of a different subclass, murine and human GM-CSF, has been purified and the cDNAs have been cloned. This protein was shown to be distinct from other CSFs, e.g., CSF-1, by Gough, et al, *Nature* (1984) 309:763-767. This GM-CSF protein is further described in W087/02060, published 9 April 1987 as being useful to treat cancer patients to regenerate leukocytes after traditional cancer treatment, and to reduce the likelihood of viral, bacterial, fungal and parasitic infection, such as acquired immune deficiency syndrome (AIDS). Murine IL-3 has been cloned by Fung, M. C., et al, *Nature* (1984) 307:233. See also Yokota, T., et al, *Proc Natl Acad Sci (USA)* (1984) 81:1070-1074; Wong, G.G., et al, *Science* (1985) 228:810-815; Lee, F., et al, *Proc Natl Acad Sci (USA)* (1985) 82:4360-4364; and Cantrell, M.A., et al, *Proc Natl Acad Sci (USA)* (1985) 82:6250-6254.)

**[0008]** Treatment of patients suffering from AIDS with CSF-1, alone or together with erythropoietin and/or an antiviral agent and/or IL-2 is reported in W087/03204, published 4 June 1987. U.S. Patent No. 4,482,485, issued 13 November 1984 states that CSF can be used for a supporting role in the treatment of cancer. In addition, EP 118,915, published 19 September 1984 reports production of CSF for preventing and treating granulocytopenia and macrophagocytopenia in patients receiving cancer therapy, for preventing infections, and for treating patients with implanted bone marrow. In addition, CSF-1 is reported to stimulate nonspecific tumoricidal activity (Ralph et al, *Immunobiol* (1986) 172:194-204). Ralph et al, *Cell Immunol* (1983) 76:10-21 reported that CSF has no immediate direct role in activation of macrophages for tumoricidal and microbiocidal activities against fibrosarcoma 1023, lymphoma 18-8, and *L. tropica* amastigotes. Ralph et al, *Cell Immunol* (1987) 105:270-279 reports the added tumoricidal effect of a combination of CSF-1 and lymphokine on murine sarcoma TUS targets.

**[0009]** In addition, Warren et al, *J Immunol* (1986) 137:2281-2285 discloses that CSF-1 stimulates monocyte production of interferon, TNF and colony stimulating activity. Lee et al, *J Immunol* (1987) 138:3019-3022 discloses CSF-1-induced resistance to viral infection in murine macrophages.

Summary of the Invention

**[0010]** The invention relates to use of recombinant colony stimulating factor 1 (CSF-1) in the manufacture of a medicament useful for therapeutic treatment of viral infectious disease in mammals, the virus being cytomegalovirus.

**[0011]** In addition, the invention relates to uses of these pharmaceutical compositions comprising CSF-1 in conjunction with one or more lymphokines selected from interferon-alpha, interferon-beta, interferon-gamma, IL-2, TNF, IL-1, GM-CSF, or G-CSF.

**[0012]** A further aspect of the invention is the use of CSF-1 in the manufacture of a medicament for therapeutic treatment of leukopenia.

**[0013]** Yet a further aspect of the invention is the use of CSF-1 in the manufacture of a medicament for enhancing the production of white blood cells.

Brief Description of the Drawings

**[0014]**

Figure 1 shows the DNA and deduced amino acid sequences for a cDNA clone encoding CSF-1.

Figure 2 shows the sequenced portion of a 3.9 kb HindIII fragment encoding human CSF-1 sequences and the deduced amino acid sequences for the exon regions.

A. Definitions

**[0015]** "Colony stimulating factor-1 (CSF-1)" refers to a protein which exhibits the spectrum of activity understood in the art for CSF-1 --i.e., when applied to the standard in vitro colony stimulating assay of Metcalf, D., J Cell Physiol (1970) 76:89, it results in the formation of primarily macrophage colonies. Native CSF-1 is a glycosylated dimer; dimerization may be necessary for activity. Contemplated within the scope of the invention and within the definition of CSF-1 are both the dimeric and monomeric forms. The monomeric form may be converted to the dimer by in vitro provision of intracellular conditions, and the monomer is per se useful as an antigen to produce anti-CSF-1 antibodies.

**[0016]** There appears to be some species specificity: Human CSF-1 is operative both on human and on murine bone marrow cells; murine CSF-1 does not show activity with human cells. Therefore, "human" CSF-1 should be positive in the specific murine radioreceptor assay of Das, S.K., et al, Blood (1981) 58:630, although there is not necessarily a complete correlation. The biological activity of the protein will generally also be inhibited by neutralizing antiserum to human urinary CSF-1 (Das, S.K., et al, supra). However, in certain special circumstances (such as, for example, where a particular antibody preparation recognizes a CSF-1 epitope not essential for biological function, and which epitope is not present in the particular CSF-1 mutein being tested) this criterion may not be met.

**[0017]** Certain other properties of CSF-1 have been recognized more recently, including the ability of this protein to stimulate the secretion of series E prostaglandins, interleukin-1, and interferon from mature macrophages (Moore, R., et al, Science (1984) 223:178). The mechanism for these latter activities is not at present understood, and for purposes of definition herein, the criterion for fulfillment of the definition resides in the ability to stimulate the formation of monocyte/macrophage colonies using bone marrow cells from the appropriate species as starting materials, under most circumstances (see above) the inhibition of this activity by neutralizing antiserum against purified human urinary CSF-1, and, where appropriate for species type, a positive response to the radioreceptor assay. (It is known that the proliferative effect of CSF-1 is restricted to cells of mononuclear phagocytic lineage (Stanley, E.R., The Lymphokines (1981), Stewart, W.E., II, et al, ed, Humana Press, Clifton, NJ), pp. 102-132) and that receptors for CSF-1 are restricted to these cell lines (Byrne, P.V., et al, Cell Biol (1981) 91:848) and placental trophoblasts).

**[0018]** As is the case for all proteins, the precise chemical structure depends on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular protein may be obtained as an acidic or basic salt, or in neutral form. All such preparations which retain their activity when placed in suitable environmental conditions are included in the definition. Further, the primary amino acid sequence may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like, more commonly by conjugation with saccharides. The primary amino acid structure may also aggregate to form complexes, most frequently dimers. Indeed, native human urinary CSF-1 is isolated as a highly glycosylated dimer. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced in vitro. In any event, such modifications are included in the definition so long as the activity of the protein, as defined above, is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the protein in the various assays.

**[0019]** Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other deriva-

tization, and the protein may be cleaved to obtain fragments which retain activity. Such alterations which do not destroy activity do not remove the protein sequence from the definition.

**[0020]** Modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence which falls within the definition of proteins "having an amino acid sequence substantially equivalent to that of CSF-1". Indeed, human- and murine-derived CSF-1 proteins have nonidentical but similar primary amino acid sequences which display a high homology.

**[0021]** For convenience, the mature protein amino acid sequence of the monomeric portion of a dimeric protein shown in Figure 1, deduced from the cDNA clone illustrated herein, is designated mCSF-1 (mature CSF-1). Figure 1 shows the presence of a 32 residue putative signal sequence, which is presumably cleaved upon secretion from mammalian cells; mCSF-1 is represented by amino acids 1-224 shown in that figure. Specifically included in the definition of human CSF-1 are muteins which monomers and dimers are mCSF-1 and related forms of mCSF-1, designated by their differences from mCSF-1. CSF-1 derived from other species may fit the definition of "human" CSF-1 by virtue of its display of the requisite pattern of activity as set forth above with regard to human substrate.

**[0022]** Also for convenience, the amino acid sequence of mCSF-1 will be used as a reference and other sequences which are substantially equivalent to this in terms of CSF-1 activity will be designated by referring to the sequence shown in Figure 1. The substitution of a particular amino acid will be noted by reference to the amino acid residue which it replaces. Thus, for example, ser<sub>90</sub>CSF-1 includes the protein which has the sequence shown in Figure 1 except that the amino acid at position 90 is serine rather than cysteine. Deletions are noted by a  $\nabla$  followed by the number of amino acids deleted from the N-terminal sequence, or by the number of amino acids remaining when residues are deleted from the C-terminal sequence, when the number is followed by a minus sign. Thus,  $\nabla$ 4CSF-1 includes the CSF-1 of Figure 1 wherein the first 4 amino acids from the N-terminus have been deleted;  $\nabla$ 130- refers to CSF-1 wherein the last 94 amino acids following amino acid 130 have been deleted. Illustrated below are a number of CSF-1 proteins, for example, asp<sub>59</sub>CSF-1, which contains an aspartic residue encoded by the gene (Figure 1) at position 59 rather than the tyrosine residue encoded by the cDNA, and  $\nabla$ 158-CSF-1 (hereinafter 158), which comprises only amino acids 1-158 of mCSF-1.

**[0023]** "Operably linked" refers to juxtaposition such that the normal function of the components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences.

**[0024]** "Control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences which are suitable for procaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood, sequences. Eucaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

**[0025]** "Effective amount" signifies an amount effective to perform the function specified, such as to kill tumors or reduce tumor burden or prevent or cure infectious diseases.

**[0026]** "Therapeutic treatment" indicates treating after the disease is contracted, whereas "prophylactic" treatment indicates treating before the disease is contracted.

**[0027]** "Mammals" indicates any mammalian species, and includes rabbits, mice, dogs, cats, primates and humans, preferably humans.

**[0028]** "Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. In order to effect transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome.

**[0029]** As used herein "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny which have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

## B. General Description

**[0030]** The CSF-1 proteins used according to the invention are capable both of stimulating monocyte-precursor/macrophage cell production from progenitor marrow cells, thus enhancing the effectiveness of the immune system, and of stimulating such functions of these differentiated cells as the secretion of lymphokines in the mature macrophages.

**[0031]** In general, any subject suffering from availability of CSF-1 for pharmacological use. In addition, subjects could be supplied enhanced amounts of previously differentiated macrophages to supplement those of the indigenous sys-

tem, which macrophages are produced by *in vitro* culture of bone marrow or other suitable preparations followed by treatment with CSF-1. These preparations include those of the patient's own blood monocytes, which can be so cultured and returned for local or systemic therapy.

**[0032]** The ability of CSF-1 to stimulate production of lymphokines by macrophages and to enhance their ability to kill target cells also makes CSF-1 directly useful in treatment of infections.

**[0033]** CSF-1 stimulates the production of interferons by murine-derived macrophage (Fleit, H.B., et al, *J Cell Physiol* (1981) 108:347), and human, partially purified, CSF-1 from MIAPaCa cells stimulates the poly(I):poly(C)-induced production of interferon and TNF from human monocytes as illustrated in co-owned PCT publication WO 86/04607, published 14 August 1986. In addition, CSF-1 stimulates the production of myeloid CSF by human blood monocytes.

**[0034]** Moreover, CSF-1 can be employed in conjunction with another efficacious agent, including lymphokines or cytokines such as, e.g.,  $\alpha$ -IFN,  $\beta$ -IFN,  $\gamma$ -IFN, IL-2, or TNF to treat tumors.

**[0035]** Also illustrated below is a demonstration of the ability of CSF-1 (from murine L-cell-conditioned medium and *E. coli* produced human recombinant CSF-1) to stimulate normal C3H/HeN mouse peritoneal macrophages to kill murine sarcoma TU5 targets. This activity is most effective when the CSF-1 is used as pretreatment and during the effector phase. The ability of CSF-1 to do so is much greater than that exhibited by other colony stimulating factors, as shown in Figure 3 hereinbelow.

**[0036]** CSF-1 may also be employed to augment the lymphokine-induced antibody-dependent cellular cytotoxicity (ADCC) of macrophages against tumor cells. This activity is particularly effective when CSF-1 is used in combination with IL-2, IFN- $\alpha$ , IFN- $\beta$  or IFN- $\gamma$ .

**[0037]** The ability of murine cells to attack infectious organisms, including viruses such as cytomegalovirus (CMV), is enhanced by CSF-1. (Murine CSF-1 is inconsistently reported to stimulate murine macrophage to be cytostatic to P815 tumor cells (Wing, E.J., et al, *J Clin Invest* (1982) 69:270) or not to kill other leukemia targets (Ralph, P, et al, *Cell Immunol* (1983) 76:10). Nogawa, R.T., et al, *Cell Immunol* (1980) 53:116, report that CSF-1 may stimulate macrophage to ingest and kill yeast.)

**[0038]** Thus, CSF-1 can be used to destroy the invading organisms indirectly by stimulation of macrophage secretions and activity.

**[0039]** CSF-1 may also be used to cure leukopenia, a disease involving a deficiency in the total number of white blood cells. Neutropenia reflects a deficiency affecting principally the polymorphonuclear leukocytes (neutrophils, granulocytes) and may be due to various infections, certain drugs (e.g., cytotoxic drugs) or ionizing radiations. Thus, *in vivo* administration of CSF-1 can be used to induce stem cells to increase the count of white blood cells, including neutrophils.

**[0040]** The CSF-1 used in the invention may be formulated in conventional ways standard in the art for the administration of protein substances. Administration by injection is preferred; formulations include solutions or suspensions, emulsions, or solid composition for reconstitution into injectables. Suitable excipients include, for example, Ringer's solution, Hank's solution, water, saline, glycerol, dextrose solutions, and the like. In addition, the CSF-1 of the invention may be preincubated with preparations of cells in order to stimulate appropriate responses, and either the entire preparation or the supernatant therefrom introduced into the subject. As shown hereinbelow, the materials produced in response to CSF-1 stimulation by various types of blood cells are effective against desired targets, and the properties of these blood cells themselves to attack invading viruses or neoplasms may be enhanced. The subject's own cells may be withdrawn and used in this way, or, for example, monocytes or lymphocytes from another compatible individual employed in the incubation.

**[0041]** The complete coding sequence for human CSF-1 is now available and expression vectors applicable to a variety of host systems have been constructed and the coding sequence expressed. See, for example, PCT publication WO 86/04607, *supra*; Kawasaki, E.S., et al *Science* (1985) 230:291-296; Ladner et al, (1987) *EMBO J* 6(9):2693-2698, WO 88/03173, published 5 May 1988, and EP 0,272,779, published 29 June 1988.

**[0042]** The variety of hosts available, along with expression vectors suitable for such hosts, permits a choice among posttranslational processing systems, and of environmental factors providing conformational regulation of the protein thus produced. Thus, the availability of this information provides the CSF-1 protein in sufficient quantity for application in the various therapies discussed herein.

**[0043]** In the particular case of human CSF-1, evidence now accumulating indicates that considerable deletion at the C-terminus of the protein may occur under both recombinant and native conditions, and that the activity of the protein is still retained. It appears that the native proteins isolated may be in some sort of C-terminal truncated form or mixtures thereof, and may exhibit variable C-terminal processing. The activity of these "truncated" forms is clearly established by their deliberate production. The mutein produced from DNA encoding SCSF/C $\nabla$ 150 (hereinafter 150), for example, is fully active in assays for CSF-1, as is that produced from cDNA encoding LCSF/C $\nabla$ 190 (hereinafter 190), LCSF/C $\nabla$ 221 (hereinafter 221), asp<sub>59</sub>LCSF/N $\nabla$ 3C $\nabla$ 22, (hereinafter N $\nabla$ 3C $\nabla$ 221). These muteins are described in copending European Publication No. 272,779, published 29 June 1988, and PCT publication WO 88/03173, published 5 May 1988.

C. Preferred Embodiments

**[0044]** The recombinant CSF-1 be considered a set of muteins which have similar but not necessarily identical primary amino acid sequences, all of which exhibit, or are specifically cleavable to a mutein which exhibits, the activity pattern characteristic of CSF-1--i.e., they are capable of stimulating bone marrow cells to differentiate into monocytes, preponderantly, and, within the limitations set forth in the Definitions section above, are immunoreactive with antibodies raised against native CSF-1 and with the receptors associated with CSF-1 activity. Certain embodiments of these muteins are, however, preferred.

**[0045]** The primary sequences shown in Figure 1 and in Figure 4 of Ladner et al, *supra*, for mature CSF-1 have the required activity, and are, of course, among the preferred embodiments. Also preferred are muteins wherein certain portions of the sequence have been altered by either deletion of, or conservative substitution of, one or more amino acids in mature CSF-1. By a "conservative" amino acid substitution is meant one which does not change the activity characteristics of the protein, and in general is characterized by chemical similarity of the side chains of the two residues interchanged. For example, acidic residues are conservatively replaced by other acidic residues, basic by basic, hydrophobic by hydrophobic, bulky by bulky, and so forth. The degree of similarity required depends, of course, on the criticality of the amino acid for which substitution is made, and its nature. Thus, in general, preferred substitutions for cysteine residues are serine and alanine; for aspartic acid residues, glutamic acid; for lysine or arginine residues, histidine; for leucine residues, isoleucine, or valine; for tryptophan residues, phenylalanine or tyrosine; and so forth.

**[0046]** Regions of the CSF-1 protein which are most tolerant of alteration include those regions of known low homology between human and mouse species (residues 15-20 and 75-84); regions which confer susceptibility to proteolytic cleavage (residues 51 and 52 and residues 191-193); cysteine residues not participating in disulfide linkages, or other residues which are not absolutely essential for activity (residues 159-224). It also appears residues 151-224 are not essential.

**[0047]** Therefore, particularly preferred are those CSF-1 muteins characterized by the deletion or conservative substitution of one or more amino acids and/or one or more sequences of amino acids between positions 159 and 224 inclusive of mCSF-1 or positions 151-224 inclusive. In particular,  $\nabla$ 158CSF-1 has CSF activity comparable to that of the native protein, even if a limited number of additional amino acid residues not related to CSF-1 are included at the truncated C-terminus;  $\nabla$ 150CSF-1 has similar activity. Indeed, the native protein is reported to have a molecular weight of 14-15 kd (as opposed to the 26 kd predicted from the cDNA sequence) and the hydrophobicity deduced from the recombinant (predicted) amino acid sequence corresponds to a transmembrane region normally susceptible to cleavage. It may, therefore, be that the truncated version corresponds in a rough way to the CSF-1 as isolated.

**[0048]** Also preferred are CSF-1 proteins which comprise the amino acid sequences containing the first 3-150 or 4-150 amino acids of SCSF or LCSF and the C-terminal deletions, such as, LCSF/C $\nabla$ 221 and asp<sub>59</sub>LCSF/N $\nabla$ 3C $\nabla$ 221.

**[0049]** Also preferred are muteins characterized by the deletion or conservative substitution of one or more of the amino acids at positions 51 and 52 and/or positions 191, 192 and 193 of mCSF-1. Especially preferred is gln<sub>52</sub>CSF-1; a corresponding proline substitution is not conservative, and does not yield an active CSF. Since they represent regions of apparently low homology, another preferred set of embodiments is that characterized by the deletion or conservative substitution of one or more of the amino acids at positions 15-20 and/or positions 75-84 of mCSF-1. Also preferred are those muteins characterized by the deletion or conservative substitution of the cysteine residue at any position not essential for disulfide bond formation. Also preferred are those muteins characterized by the deletion or substitution of the tyrosine residue at position 59 of mCSF-1; particularly, substitution by an aspartic acid residue.

D. Cloning and Expression of Human CSF-1

**[0050]** PCT publication WO 86/04607, *supra*, illustrates the methods used in obtaining the coding sequence for human CSF-1, for disposing this sequence in expression vectors, and for obtaining expression of the desired protein. The teaching of this publication is specifically incorporated herein by reference.

**[0051]** The full length cDNA is 1.64 kb and encodes a mature CSF-1 protein of 224 amino acids. The clone was designated CSF-17 with Cetus depository number CMCC 2347 and was deposited with the ATCC (ATCC 53149) on 14 June 1985. The plasmid bearing the CSF-1 encoding DNA was designated pcCSF-17.

D.1 Mutein-Encoding Sequences

**[0052]** Modifications were made of the pcCSF-17 inserts to provide corresponding plasmids encoding muteins of the mCSF-1 protein. For site-specific mutagenesis, pcCSF-17 and M13mp18 were digested with the same restriction enzyme excising the appropriate region of the CSF-1 coding sequence, and the excised sequence was ligated into the M13 vector. Second strand synthesis and recovery of the desired mutated DNA use the following oligonucleotide primers:

for pro<sub>52</sub>CSF-1, 5'-TACCTTAAACCGGCATTCTC-3', which creates a new HpaII site at codons 52-53;  
 for gln<sub>52</sub>CSF-1, 5'-TACCTTAAACAGGCCTTCTC-3', which creates a new StuI site at codons 52-53;  
 for asp<sub>59</sub>CSF-1, 5'-GGTACAAGATATCATGGAG-3', which creates a new EcoRV site at codons 59-60.

**[0053]** After second strand extension using Klenow, the phage were transformed into *E. coli* DG98 and the resulting plaques screened with kinased labeled probe. After plaque purification, the desired mutated inserts were returned to replace the unmutated inserts in pCCSF-1, yielding PCSF-pro<sub>52</sub>, pCSF-gln<sub>52</sub>, and pCSF-asp<sub>59</sub>, respectively.

**[0054]** Plasmids containing three deletion mutants which encode  $\nabla$ 158CSF-1 were also prepared: pCSF-Bam, pCSF-BamBcl, and pCSF-BamTGA. For pCSF-Bam, pcCSF-17 was digested with BamHI, and the upstream BamHI/BamHI fragment of the coding region was isolated and religated to the vector fragment. The ligation mixture was transformed into *E. coli* MM294 and plasmids with the correct orientation were isolated. The resulting pCSF-Bam encodes 158 amino acids of the CSF-1 protein fused to six residues derived from the vector at the C-terminus: Arg-His-Asp-Lys-Ile-His.

**[0055]** For pCSF-BamBcl, which contains the entire CSF-1 encoding sequence, except that the serine at position 159 is mutated to a stop codon, the coding sequence was excised from pcCSF-17 and ligated into M13 for site-specific mutagenesis using the primer: 5'-GAGGGATCCTGATCACCGCAGCTCC-3'. This results in a new BclI site at codons 159-160. The mutated DNA was excised with BstXI/EcoRI and ligated into the BstXI/EcoRI digested pcCSF-17, the ligation mixture was transformed into *E. coli* DG105, a dam<sup>-</sup> host, and the plasmid DNA isolated.

**[0056]** For pCSF-BamTGA, in which the codons downstream of the 159-stop are deleted, pCSF-BamBcl was digested with XhoI and BclI, and the insert ligated into XhoI/BamHI digested pcCSF-17.

**[0057]** In addition, PCSF-Gly<sub>150</sub>, which contains a TGA stop codon instead of histidine at position 151, was prepared from the pcCSF-17 insert by site-specific mutagenesis using the appropriate primer, as described above.

## D.2 Expression and Biological Assay of CSF-1

**[0058]** The expression of plasmid DNA from CSF-17 (pcCSF-17) in COS-7 cells was confirmed and quantitated using the bone marrow proliferation assay, the colony stimulation assay and the radioreceptor assay as taught in WO 86/04607, *supra*. It will be recalled that the specificity of the bone marrow proliferation assay for CSF-1 resides only in the ability of CSF-1 antiserum to diminish activity; that for the colony stimulation assay, in the nature of the colonies obtained. Both assays showed CSF-1 production to be of the order of several thousand units per ml.

## D.3 Expression of Muteins

**[0059]** In a similar manner to that taught for pcCSF-17, the mutein-encoding plasmids were transfected into COS-A2 cells and transient expression of CSF-1 activity was assayed by the bone marrow proliferation assay and by radioimmunoassay using anti-CSF antibodies. The expression product of pCSF-pro52 was inactive, indicating that, as expected, substitution by proline is not conservative. All other muteins showed activity in both assays as shown by the results below:

Expression of CSF-1 Constructs in COS Cells			
CSF-1 Plasmid	Radio- immunoassay	Bone Marrow Assay (units/ml)	
		Proliferation	Colony
	(units/ml)	(units/ml)	
pcCSF-17	3130	2798	11,100
	3080	3487	9750
	3540	3334	11,500
pCSF-pro52	54.8	<25	<100
	51.9	<25	<100
	45.3	<25	<100
pCSF-gln52	1890	2969	6200
	2250	2308	5500
	1910	2229	4400

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(continued)

Expression of CSF-1 Constructs in COS Cells			
CSF-1 Plasmid	Radio- immunoassay	Bone Marrow Assay (units/ml)	
		Proliferation	Colony
	(units/ml)	(units/ml)	
pCSF-asp59	3210	3381	9000
	4680	3417	6800
	3470	2812	10,600
pCSF-Bam	9600	8048	22,600
	8750	8441	21,900
	8400	10,995	21,700
pCSF-BamBcl	8800	--	26,000
	10,700	--	21,600
	15,450	--	24,200
pCSF-BamTGA	8450	--	22,600
	7550	--	23,200
	9700	--	20,000
pCSF-Gly150	26,850	--	55,710

### D.4 Murine CSF-1

**[0060]** An intronless DNA sequence encoding murine CSF-1 is taught in WO 86/04607, supra

**[0061]** This murine CSF-1 is prepared using a murine fibroblast cell line which produces large amounts of CSF-1. The L-929 line, obtainable from ATCC, is used as a source for mRNA in order to produce a cDNA library. Using oligo-  
 meric probes constructed on the basis of the known murine N-terminal and CNBr-cleaved internal peptide sequence, this cDNA library is probed to retrieve the entire coding sequence for the murine form of the protein. Murine CSF-1 is believed to be approximately 80% homologous to the human material because of the homology of the N-terminal sequences, the ability of both human and murine CSF-1 preparations to stimulate macrophage colonies from bone marrow cells, and limited cross-reactivity with respect to radioreceptor and radioimmunoassays (Das, S. K., et al, Blood (1981) 58:630). The preparation of the cDNA, cloning and expression of murine CSF-1 are as described in WO 86/04607, supra.

### E. Activity of CSF-1

**[0062]** The activity of CSF-1 was determined in the following Examples using partially purified MIAPaCa CSF-1, murine L cell CSF-1, CV-1-produced recombinant material or E. coli-produced human CSF-1.

**[0063]** CSF-1 was demonstrated to inhibit the growth of cytomegalovirus in mice.

**[0064]** The following Examples are illustrative, not limiting

### Examples

#### In Vitro Stimulation of Murine Antiviral Activity

**[0065]** Adherent murine thioglycolate-elicited macrophages were incubated with CSF-1 for 3 days and infected with VSV overnight (Lee, M.T., et al (1987) J Immunol 138:3019-3022). The following table shows crystal violet staining as measured by absorbance at 550 nm of cells remaining adherent.



Treatment	Absorbance (mean)(S.D.)
Medium/No virus	0.346±0.02
Medium + virus	0.170±0.02
CSF-1, 1000 U/ml + virus	0.264±0.02
CSF-1, 2000 U/ml + virus	0.356±0.04

**[0066]** CSF-1 treated cells, therefore, showed protection of the macrophage against VSV.

#### In Vivo Treatment of CMV Infection with CSF-1

**[0067]** Outbred CD-1 mice were treated with the CSF-1 (158) produced from the CV-1 cell line at doses of 400 µg/kg, intraperitoneally, once a day for five days, starting two days before infection with a sub-lethal dose of cytomegalovirus (CMV). Mice were sacrificed on the third day after infection and the extent of viral replication in target organs such as the spleen was evaluated by plaque assay. The results showed that mice treated with CSF-1 have significantly lowered (57.8% reduction in) spleen viral titer compared to the saline-treated control mice, indicating that CMV infection is less severe in CSF-1-treated mice.

**[0068]** Separately, CSF-1 produced in *E. coli* (N73C7221) has been tested in a lethal murine CMV infection model in outbred CD-1 mice (this is in contrast to the above experiment using sub-lethal doses of CMV, in which organ titers were monitored). When CSF-1 was administered intraperitoneally to mice at 3-4 mg/kg (single dose given per mouse) 24 hours before viral challenge, there was a significant increase in survival as compared to saline-treated control.

**[0069]** Thus, CSF-1 may be used alone or in combination with another lymphokine in the treatment of viral infections in general, and in particular, may be beneficial in immunosuppressive viral infection such as acquired immune deficiency syndrome (AIDS).

**[0070]** The preferred dosage range is about 0.4-4 mg/kg CSF-1 per mouse per day.

#### Leukopenic Infection Model

**[0071]** Results of CSF-1 induced protection in *E. coli* infection in mice pretreated with 50 mg/kg cyclophosphamide (CY). The LD<sub>50</sub> of CY for mice is at about 400 mg/kg, the lower CY dose we used represents 1/8 of the LD<sub>50</sub>. This dose, when injected ip 3 days earlier, induced a decrease in total white blood cells and neutrophils, and rendered mice more susceptible to *E. coli* infection (e.g., infection with 3 x 10<sup>7</sup> cfu/mouse killed 100% of CY treated mice but only 20% of mice not given this dose of CY). When CSF-1 was given to CY treated mice (CSF-1 at 0.89 mg/kg, once/day for 4 days, ip), there was 100% survival as compared to 30% in mice given saline instead.

#### In Vivo Stimulation of White Blood Cell Count

**[0072]** Outbred CD-1 mice were administered purified recombinant human CSF-1, at 2 mg/kg per dose, three times a day for five consecutive days. Total white blood cell count increased to 12,000-13,000/µl in CSF-1-treated mice from 8,700/µl in saline-treated control mice. In addition, neutrophil count increased to 6,821/µl in CSF-1-treated mice as compared to 1,078/µl in saline-treated control mice.

**[0073]** This effect is dependent on the dose of CSF-1 and the schedule of administration. The increase in peripheral blood neutrophils was detectable 2-4 hours after a single dose of CSF-1 was administered intraperitoneally. These results indicate that CSF-1 administration may be useful in clinical or veterinary medicine as a stimulus of granulocyte production and an enhancer of white blood count.

#### F. Formulation and Dosage of CSF-1

**[0074]** The recombinantly produced human CSF-1 may be formulated for administration using standard pharmaceutical procedures. Ordinarily CSF-1 will be prepared in injectable form, and may be used either as the sole active ingredient, or in combination with other proteins or other compounds having complementary or similar activity. Such other compounds may include alternate antitumor, e.g., chemotherapeutic, agents such as adriamycin, or lymphokines, such as IL-1, -2, -3, and -4, alpha-, beta-, and gamma-interferons, CSF-GM and CSF-G, and tumor necrosis factor. The effect of the CSF-1 active ingredient may be augmented or improved by the presence of such additional components. As described above, the CSF-1 may interact in beneficial ways with appropriate blood cells, and the compositions of the invention therefore include incubation mixtures of such cells with CSF-1, optionally in the presence of additional

lymphokines or cytokines. Either the supernatant fractions of such incubation mixtures, or the entire mixture containing the cells as well, may be used. Staggered timing may be preferred for some combinations, such as CSF-1 followed one to two days later by  $\gamma$ -interferon.

**[0075]** The CSF-1 described herein is generally administered therapeutically for CMV infection treatment in amounts of between 0.01-1 mg/kg per day, whether single bolus administration or fractionated over 24 hr.

The following deposits have been made:

**[0076]**

Plasmid	CMCC No.	ATCC No.	Deposit Date
pHCSF-1		40177	2 April 1985
pHCSF-1a	2312	40185	21 May 1985
CSF-17	2347	53149	14 June 1985
pLCSF221A	3095	67390	14 April 1987
pCSF-asp59	2705	67139	19 June 1986
pCSF-gln52	2708	67140	19 June 1986
pCSF-pro52	2709	67141	19 June 1986
pCSF-Bam	2710	67142	19 June 1986
pCSF-BamBcl	2712	67144	19 June 1986
pCSF-Gly150	2762	67145	19 June 1986

**[0077]** These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The deposits will be made available by ATCC under the terms of the Budapest

**[0078]** Treaty, and subject to an agreement between Applicants and ATCC which assures permanent and unrestricted availability upon issuance of the pertinent US patent. The Assignee herein agrees that if the culture on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced upon notification with a viable specimen of the same culture. Availability of the deposits is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

**[0079]** These deposits were made for the convenience of the relevant public and do not constitute an admission that a written description would not be sufficient to permit practice of the invention or an intention to limit the invention to these specific constructs. Set forth hereinabove is a complete written description enabling a practitioner of ordinary skill to duplicate the constructs deposited and to construct alternative forms of DNA, or organisms containing it, which permit practice of the invention as claimed.

## Claims

1. The use of CSF-1 for the manufacture of a medicament for the treatment of leukopenia.
2. The use as claimed in claim 1, wherein the treatment is therapeutic.
3. The use of CSF-1 for the manufacture of a medicament for enhancing production of white blood cells.
4. The use as claimed in any of claims 1 to 3, wherein the medicament is in injectable form.
5. The use as claimed in any preceding claim, wherein CSF-1 is augmented by derivatisation.
6. The use as claimed in claim 5, wherein the CSF-1 is conjugated to sugar moieties, e.g. saccharides, lipids, phosphate or acetyl groups.
7. The use as claimed in any preceding claim, wherein the medicament further comprises other proteins or compounds having complementary or similar activity.

8. The use as claimed in claim 7, wherein the other proteins or compounds are antitumor agents, e.g. chemotherapeutic agents, such as adriamycin.
- 5 9. The use as claimed in claim 7, wherein the other proteins or compounds are lymphokines, e.g. IL-1, IL-2, IL-3, IL-4,  $\alpha$ -interferon,  $\beta$ -interferon,  $\gamma$ -interferon, CSF-GM, CSF-G or tumor necrosis factor.

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FIG. 1a

20 40 60 80 100 120  
 AGTGAAGCTC GGGCCGGGGA AAGTGAAGT TTGCTGGGT CCTCTGGGG CCAGAGCCG TCTCCGATC CCAGGACAC GGTCCGGGG TCGCCACATCC GCGGACGCCA  
 140 160 180 200 220  
 CCGAGCGAAC GAGCGAGCGA GGGCGGCGCA CCGGACCCAG CTGCGCGT ATG ACC GCG CCG GCG GCG TCC CCT CCC ACG ACA TGG CTG GGC  
 Met Thr Ala Pro Gly Ala Ala Gly Arg Cys Pro Pro Thr Thr Trp Leu Gly  
 -32  
 240 260 280 300 320  
 TCC CTG CTG TTG GTG TGT CTC CTG AGG AGT ATC ACC GAG GAG GTG TCG GAG TAC TGT ACC CAC ATG ATT GGG AGT CGA CAC CTG CAG TCT  
 Ser Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu Gln Ser  
 340 360 380 400 420  
 CTG CAG CCG CTG ATT GAC AGT CAG ATG GAG ACC TCG TCG CAA ATT ACA TTT GAG TTT GTA GAC CAG GAA CAG TTG AAA GAT CCA GTG TCC TAC CTT AAG  
 Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln Ile Thr Phe Glu Phe Val Asp Gln Gln Glu Leu Lys Asp Pro Val Cys Tyr Leu Lys  
 440 460 480 500 520  
 AAG GCA TTT CTC CTG GTA CAA TAC ATA ATG GAG GAC ACC ATG CCG TTC AGA GAT AAC ACC CCC AAT CCC ATC GCC ATT GTG CAG CTG CAG GAA CTC TCT  
 Lys Ala Phe Leu Leu Val Gln Tyr Ile Met Glu Asp Thr Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu Glu Leu Ser  
 540 560 580 600 620  
 CTG AGG CTG AAG ACC TCC TTC ACC AAG GAT TAT GAA GAG CAT GAC AAG GCG TCC GTC CGA ACT TTC TAT GAG ACA CCT CTC CAG TTG CTG GAG AAG GTC  
 Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln Leu Leu Lys Val  
 640 660 680 700 720  
 AG AAT GTC TTT AAT GAA ACA AAG AAT CTC CTT GAC AAG GAC TGG AAT ATT TTC ACC AAG AAC TCC AAC AAG AGC TTT GCT GAA TCC TCC AGC CAA GGC  
 ys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala Glu Cys Ser Ser Gln Gly  
 120 140

FIG.1b

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740      CAT GAG AGG CAG TCC GAG GGA TCC ACC CCG CAG CTC CAG GAG TCT GTC TTC CAC CTG CTG CCG ACT GTC ATC CTG GTC TTG CTG GGC GTC GGA
      His Glu Arg Gln Ser Glu Gln Ser Glu Gln Ser Val Phe His Leu Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val Gly
180
840      GGC CTC TTG TTC TAC AGG TGG AGG CCG AGC CAT CAA GAG CCG CAG AGA GCG GAT TCT CCG TTG GAG CAA CCA GAG GGC AGC CCG CTG ACT CAG GAT
      Gly Leu Leu Phe Tyr Arg Trp Arg Arg Ser His Gln Glu Pro Gln Arg Ala Asp Ser Pro Leu Glu Gln Pro Gly Ser Pro Leu Thr Gln Asp
200
940      GAC AGA CAG GTG GAA CTG CCA GTG TAG AGGGAATTCTA AGACCCCTCA CCATCCTTGA CACACTCGTT TGTCAATGTC CCTCTGAAA TGTGACGCCC AGCCCCGGAC
      Asp Arg Gln Val Glu Leu Pro Val
220
1040     ACAGTACTCC AGATGTTGTC TGACCAGCTC AGAGAGAGTA CAGTGGGACT GTTACCTTCC TTGATATGGA CAGTATTCTT CTATTTGTG AGATTAAAGAT TGCATTAGTT TTTTCTTAA
1100
1160     CAACTGCATC ATACTGTTGT CATATGTTGA GCGTGTGGTC TATTAAAGCC CCTAGTTCCA TTTCCTAATA ACTTCTGTCA AGCCAGACCA TCTCTAUCCT GTACTTGGAC AACTTAACTT
1200
1280     TTTTAAACCA AGTCCAGTTT ATGTTACCT TTTTAAAGC CACCTTGTGG TTTCGTGCGA TCACCTGAAC CTACTGAAGT TGTGTGAAAT CCTAATTCGT TCACTCTCCG AGCCCTCCCA
1300
1400     GTTGTGCTC CTGCACATTG ATGAGTCCCT GCTGTGTGTCT TTCCCATGT TGTTGATGTA GCCTGTGACC TATTGTTCCT CACCCCTGCC CCCCAGCCAC CCAACCTCTC
1420
1520     CCCCCTGCCAC CCAAGCCAC AGCCAGCCCA TCAGGAAGCC TTCCCTGGCTT CTCACAAAC TTCTGACTGC TCTTTTCAGT CATGCCCTTC CTGCTCTTTT GTATTGCTT AATAGTATAT
1540
1640     CAATTTCAC TT

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PARTIAL HUMAN CSF-1 SEQUENCE

1 GAAGGTGACATCTGGGCTGTTTTTCATGGGAGAACAAAGGTTGTGCTGTGGCTGCTAGAAAT  
61 CCTGGGAAAGCTGGATTTGAGGGATGCTGTATCCTGAGGTAAGGGCAGAGCCTGTAGCAT  
121 TGTAGATATGAGGCCTTTGTTTTCTGCGTTGAGCAGGGCATGGGGATAACTGGGGAGAG  
181 TGAGACCTGGGGAGAAATGACACCCTCTCTGTACACAGACATGGCTGGGCTCCCTGCTGTT  
ThrTrpLeuGlySerLeuLeuLeu  
241 GTTGGTCTGTCTCCTGCGGAGCAGGAGTATCACCAGGAGGAGTGTCCGAGTACTGTAGGCA  
LeuValCysLeuLeuAlaSerArgSerIleThrGluGluValSerGluTyrCysSerHis  
301 CATGATTGGGAGTGGACACCTGGAGTCTCTCCAGGAGCTGGTGTAGTGTGTGGCCATGCTG  
METIleGlySerGlyHisLeuGlnSerLeuGlnArgLeu  
361 TATTCTACCTTCTCCCCACTGGGGAAATGAAGGCAGGAGCCAGGGAGCAGGTCAAAGAGA  
421 GCAGTTGCAGGCAGGAAAAATAGGGCAGTGGGGACATTGCTTGTGGTTCCTACTAGCTCC  
481 ACCAGTGATACCCTTCACTAACCTTCCCAAAGTTAGGACCTCTGGTCTCCCCAGCTCGAA  
541 GCCCTCTCTGACTGCCCTGCAGGCAGTGGATGCTGTGGGCTTCCAGCTGCTTGCTTGGGT  
601 TAGTGATTGCCCAGGAACATCAACCACTGATTCTGAAAAGGCTTCTGAGGTCTGCTGTCC  
661 CTCAGTGGGATGCCTCCTCTGGGAAGCTAGCCCAGGCGGCTGCTGTGTCCAGATGTTGC

FIG. 2a

721 ATCTAGCCTCCTGGACTCTCATATGTGGCGCASTCTGCGATCAGAGCCCCACCAGATTTG

781 GAGGGAAGCGCTTGCCTAACTCCAGCCTCCACACTCACTT . . . 200 BASES . .

. . . TCCAGGGCCCTGAGCTTGGGGCCTGTGGGCTGTGCCTTCCGCCCTCTCTTGCCCCAGCACT

61 ACTTCTCCTGTATGTAGTTGCTGTAGCAATCCAAGGTAGACCAAGAGCCCCAGCATTCTC

121 TGAGGCTTAAAATCCAGAACTGCTGCTCTGGGGCTAAAGAGGGCTTTAAGGGCATCCAGC

181 TCCAACCCCTACAGGTGTTCAATCCCCAGAGCTTGTCCAGCCTCTGCTTGAATTGCTAGC

241 ATGACAGGGTGGTCACTGCTCCAGGGAAGATTATCTATATTCTTCTATAGACATCT

301 CTTCCTTAAAACAAATGGGCATTTGTGAGATTTGTGGGGTGGTAGAAGGAAAGAGAGA

361 CTTCCTTGTCTCTTACAGCCTTCCCTGGGCATCTGGAAGGCACTGATCTTCTCTAGAC

421 TTGACTCTGTCTTTCCACGTGTGGTGGCAGGGATGAAGTTCAAACCCCAATCCACTGAG

481 AAGCTAAGGTCCCGTTTTGAAGAAGGCTGAAGGCTGAGTTGAGCTGTAGGTTACCCCTGC

541 AATCGTTGGCCTGCTCTCTCTTACAGATTGACAGTCAGATGGAGACCTCGTGCCAAATTA

IleAspSerGlnMETGluThrSerCysGlnIleThr

601 CATTTGAGTTTGTAGACCAGGAACAGTTGGTGAGTGATGGCTTTTTACAAAATCCATGCA

PheGluPheValAspGlnGluGlnLeu

FIG. 2b

661 CCAGCCTGCATGCAACTCCCAGGGTGGGGTGTGTGGGGGAGCATGAAAGCGGCAGAAATGC  
 721 CTACTGCTGGAAAGGGTGAGAGTGTGAGGATCCATGSGTGCTCAACTCTGGGGTGCCAGG  
 781 ATCCAGGGCTCAAGTCCCCTGCCATTCCCTTCTCCTGGCCTGATACATAACAAGGGCTCA  
 841 CTAGGTACCAAGCACTTTGCTAATGTAGTTCTGACASTACCACTATGTGCTACACAAATA  
 901 CAGTTTATTATCCACAGAGAGGGTGAAGAGGACATAGCTAGTAGGTGCTAGAGGGCTGATT  
 961 TGAATCCAGGAAGGTTGGCTGTAGGGCTTGAGGGCAATCAATACTTCTTCCAGGTCAATA  
 1021 GCTT

\* denotes match to murine amino acid sequence  
 — denotes match to human amino acid sequence  
 ↓ denotes intron/exon boundary

FIG. 2c